REMARKS/ARGUMENTS

Claims 1-116 are pending claims. Claims 1-38, 43, 55, 56, 59-63 and 66-95 are currently under consideration. Please add new claims 117-129. No new matter has been entered. Applicants respectfully request reconsideration in view of the following remarks and claim amendments. Issues raised in the Office Action will be addressed below in the order they appear in the Action.

1. Applicants note with appreciation that the response filed April 24, 2003 has been entered.

2. Claims 62 and 63 are objected to under 37 CFR 1.75(c) as being of improper dependent form for allegedly failing to further limit the subject matter of a previous claim. Applicants respectfully disagree. The test as to whether a claim is a proper dependent claim is that it shall include every limitation of the claim from which it depends (35 U.S.C. 112, fourth paragraph). According to MPEP 608.01(n)(III), a dependent claim is not an improper dependent claim, "simply because there is a question as to (1) the significance of the further limitation added by the dependent claim, or (2) whether the further limitation in fact changes the scope of the dependent claim from that of the claim from which it depends." Applicants submit that claims 62 and 63 include every limitation of their base claims, and as such, are in proper dependent form.

Furthermore, contrary to what the Action states, the inclusion of cytotoxic or immunogenic agents in claims 62 and 63 does not conflict with the limitations of claims 1-5. In claims 1-5, the limitation of "neither cytotoxic nor immunological mechanisms are needed for cell killing" describes an inherent property of the multivalent polypeptide. Claims 62 and 63, on the other hand, are directed to compositions of claims 1-6 linked to a cytotoxic or an immunologic agent respectively. The limitations in claims 62-63 are by no means contrary to the limitations of claims 1-6, since the former describe a component added to the compositions of claims 1-6, whereas the latter sets forth an additional inherent property of the multivalent

polypeptide itself. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

Claims 62 and 63 are additionally rejected under 35 U.S.C. 102(b) for allegedly being anticipated by Schlom and the abstract of Geroge et al., respectively. Applicants traverse this rejection. Nevertheless, solely to expedite prosecution, Applicants have amended claims 62 and 63 to be dependent upon allowable claims 22-29. Applicants submit that the amendments overcome the rejection. Accordingly, Applicants submit that claims 62 and 63, as amended, are now in condition for allowance.

3. Claim 70 is objected to for being of improper dependent form for failing to further limit the subject matter of a previous claim, namely, claim 67. Applicants have amended claim 70 and submit that as amended, claim 70 is now in condition for allowance.

4. Applicants note with appreciation that the Examiner deemed claims 24-29 and 82-87 allowable if rewritten to include all of the limitations of the base claims and all intervening claims. Applicants have rewritten these claims accordingly and submit that they are now in condition for allowance.

5. Claims 13-16, 22, 23, 33, 67, 80 and 81 are rejected under 35 U.S.C. 112, second paragraph, for allegedly being indefinite for failing to particularly pointing out and distinctively claiming the subject matter which Applicants regards as the invention. Applicants traverse this rejection to the extent it is maintained in light of the claim amendments.

A. Specifically, the Examiner alleged that claims 13-16 are "vague and indefinite in the recitation of GRANTA-519, KARPAS-422, LG-2 and PRIESS as the only means of identifying the cell lines" used in the invention. (See page 5 of the Action).

Applicants note that all four cell lines recited are widely known and readily available. Among the four cell lines at issue, three are available through a public depository. Specifically, GRANTA-519, a human B cell lymphoma cell line, is available through Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the accession number ACC 342. KARPAS-422, a human T cell lymphoma cell line, is also available through DSMZ under the accession number ACC 32. PRIESS, a human B lymphoblastoid cell line, is available through European Collection of Cell Cultures (ECACC), under the accession number 86052111. The fourth cell line, LG-2, a human B lymphoblastoid cell line, has been known for a long time and is widely distributed. LG-2 is widely used in research studies. In fact, Nagy et al. (WO96/17874), an application cited extensively by the Examiner in the present Office Action, used LG-2 in their studies. See Examples 5, 8 and 15 of the WO96/17874 application. To further show the wide distribution of LG-2, a sample list of scientific articles that cited the use of LG-2 is attached as Exhibit 1. Therefore, although no accession number is currently available for LG-2 cell line, one of ordinary skill in the art understands its meaning and can easily obtain LG-2 cell line.

The Examiner noted that amending claims 13-16 to incorporate a deposit accession number for each of the cell lines listed would overcome this rejection. Accordingly, Applicants have amended claims 13-16 to incorporate accession numbers wherever possible. In addition, solely to expedite prosecution, Applicants have further amended claims 13-16 to be dependent upon allowable claims 22-29. Applicants submit that they are now in condition for allowance.

B. Claims 22, 23, 67, 80 and 81 are rejected for allegedly being vague and indefinite in reciting the term MS-GPC-8-27-41 to identify the clone. The Examiner noted that amending claims 22, 23, 67, 80 and 81 to incorporate a sequence identification number for MS-GPC-8-27-41 would overcome this rejection. Applicants have amended the claims as suggested.

Regarding the non-elected clones in these claims, Applicants have consulted with the Examiner during a telephonic interview on January 5, 2004. The Examiner indicated that the non-elected clones may now be included. As Applicants pointed out during the interview, although the VH and VL sequences for ten of the clones listed in the claims were not explicitly

described in the specification, a person of ordinary skill in the art could easily determine these sequences. As described in the specification, these ten clones were derived during optimization rounds from the clones for which VH and VL sequences were explicitly described. For example, MS-GPC-8-1, MS-GPC-8-9 and MS-GPC-8-18 were derived from MS-GPC-8 during the first round of optimization, with the only differences residing in their VL-CDR3 regions (see page 46-49 of the specification). Likewise, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, and MS-GPC-8-6-47 were derived from MS-GPC-8-6, and MS-GPC-8-27-7 and MS-GPC-8-27-10 were derived from MS-GPC-8-27, with the only differences residing in their VL-CDR1 regions (see page 50-51 of the specification) respectively. The VH and VL sequences for the starting clones, MS-GPC-8, MS-GPC-8-6 and MS-GPC-8-27, were fully disclosed in the specification (see Figure 15). Together with the sequences of VL-CDR1 and VL-CDR3 for each of these clones disclosed in Table 1 of the specification (see page 77-78 of the specification), one of ordinary skill could determine the VH and VL sequences of these clones at the time of the filing of the present application. For clarity, Applicants have amended the specification to include the VH and VL sequences for these ten clones (see Figure 15 as amended). The Examiner agreed to this approach at the telephonic interview. No new matter has been entered. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Claims 13-16, 22, 23, 33, 67, 80 and 81 are further rejected under 35 U.S.C. 112, second paragraph, for allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. For reasons stated above, Applicants traverse this rejection to the extent it is maintained in light of the amended claims. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested. Applicants submit that claims 13-16, 22, 23, 67, 80 and 81, as amended, are now in condition for allowance.

C. Claim 33 is rejected for allegedly being vague and indefinite in the recitation of "a mini-antibody fragment" without a definition. See page 5 of the Office Action. Applicants respectfully point out that, contrary to the Examiner's assertion, the specification provided a definition for the term. Lines 17-22 on page 25 of the specification states:

> "As used herein, the term 'mini-antibody fragment' means a multivalent antibody fragment comprising at least two antigen-binding domains multimerized by self-associating domains fused to each of said domains (Pack, 1994), e.g. dimers comprising two scFv fragments, each fused to a self-associating dimerization domain. Dimerization domains, which are particularly preferred, include those derived from a leucine zipper (Pack and Plückthun, 1992) or helix-turn-helix motif (Pack et al., 1993)."

Accordingly, Applicants submit that the recitation of "a mini-antibody fragment" is not vague and indefinite. Nevertheless, solely to expedite prosecution, Applicants have amended claim 33 to be dependent upon allowable claims 22-29. Support for the amendments can be found throughout the application, and no new matter has been entered. Accordingly, Applicants submit that claim 33, as amended, is now in condition for allowance.

6. Applicants add new claims 117-125 as dependent claims of one or more of allowable claims. As a dependent claim, each of these new claims includes all of the limitations of the respective allowable claims. Support for these dependent claims can be found throughout the specification. No new matter was introduced by adding these dependent claims. Accordingly, Applicants submit that the new claims 117-125 are in condition for allowance.

7. Claim 17 and 38 are rejected under 35 U.S.C. 112, second paragraph, for allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. Applicants traverse this rejection to the extent it is maintained in light of the claim amendment.

First, Applicants note that the Examiner did not a state reason(s) for rejecting claim 17. See page 5-7 of the Office Action. Applicants submit that support for claim 17 can be found on page 14, lines 16-20 of the specification. Clarification is respectfully requested.

Second, Applicants have amended claim 38 to obviate the rejection. Claim 38, as amended, is directed to a composition of claims 1-6, wherein the antigen-binding domain is cross-linked to a polymer. The specification provides examples of how to achieve this without destroying the binding activity of the antigen binding domain. For example, in Example 8 of the

specification, two anti-HLA-DR antibody fragments, each of which has its V_H chain fused to a FLAG epitope, were cross-linked to each other via an anti-FLAG antibody. This was accomplished by incubating the anti-HLA-DR antibody fragments together with anti-FLAG M2 monoclonal antibody (see page 58 of the specification).

Accordingly, reconsideration and withdrawal of the objection is respectfully requested. Solely to expedite prosecution, Applicants have further amended claims 17 and 38 to be dependent upon allowable claims 22-29. Support for the amendments can be found throughout the application, and no new matter has been entered. Accordingly, Applicants submit that claims 17 and 38, as amended, are now in condition for allowance.

For the remaining rejections, for clarity and for the convenience of the Examiner, Applicants will first address the rejections for the independent claims, and next address the rejections for the dependent claims.

8. Claims 2 and 66 are rejected under 35 U.S.C. 102(b) for allegedly being anticipated by Nagy et al. Applicants traverse this rejection.

The standard for anticipation is that "a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single reference reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

Claims 2 and 66 both contain a limitation that the antigen-binding domain binds to a human HLA-DR "with a Kd of 1 μM or less". Applicants submit that this limitation is not described, either expressly or inherently, in Nagy. Prior to the filing date of this application, antibodies that binds to human MHC class II antigen with a Kd of 1μM or less had not been disclosed. In the present application, Applicants have produced antibodies with better affinities for the human HLA-DR than the antibodies disclosed in the prior art. For example, the IgG forms of certain anti-HLA-DR antibody fragments have affinities in the sub-nanomolar range.

Fab fragments affinity maturated at only the CDR3 light chain region showed affinities in the range of 40-100 nM. Even the Fab fragments of non-optimized antigen binding domains showed affinities in the sub-μM range. See Example 7 and Table 3.

Nevertheless, solely to expedite prosecution of the remaining claims, Applicants hereby cancel claims 2 and 66. Applicants reserve the right to pursue claims of similar scope in a future application.

9. Claims 1-5, 66 and 68 are rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Nagy et al as evidenced by Drenou et al and Abbas et al., and Engberg et al., in view of Winter et al. and Ames et al. Applicants traverse this rejection to the extent it is maintained in light of the claim amendments.

To establish *prima facie* obviousness of a claimed invention, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, a reasonable expectation of success is required. Finally, all the claim limitations must be taught or suggested by the prior art. See MPEP 2143. Applicants submit that, at the time of the filing of the present application given the state of the art and the level of skill in the art, a reasonable expectation of success for producing antibodies as presently claimed was absent.

Each of the independent claims 1, 3-5 and 68 contains a limitation of "an antibody-based antigen-binding domain *of human composition*". None of the cited references, either singly or in combination, teaches an antibody with such a limitation. Nagy, the principal reference cited by the Examiner, described only murine anti-HLA-DR antibody fragments. Murine monoclonal antibodies are known to elicit an undesirable host immune response that limits their efficacy. Schlom suggested humanization as an alternative to address the problem with murine antibodies, whereas Winter et al. and Engberg et al. described procedures for screening libraries of human antibody fragments.

Applicants submit that the combination of the references cited is insufficient to establish that a reasonable expectation of success for producing anti-HLA-DR antibodies of human compositions existed at the time of filing. Mere procedures for screening libraries of human antibody fragments, as described by Winter and Engberg, did not give one of ordinary skill in the art a reasonable expectation of success. Many variables could impact the results of screening, making the screening outcome highly uncertain. Examples of such variables include: diversity of the library, antigen used for screening, selection stringency, etc. At the time of filing, only a limited number of human antibodies of desired specificity and affinity had been successfully obtained, highlighting the technical difficulties in this field. Indeed, one reviewer commented that, despite the techniques of making humanized antibodies, "major problems remain" with antibody therapeutics. See Martin J.S. Dyer, Seminars on Oncology, 26: 52-57, at 52, top of right column (1999), a copy of which was submitted as document number AI with the Information Disclosure Statement on Feburary 3, 2003.

Furthermore, an applicant may rebut a *prima facie* case of obviousness pursuant to MPEP 2144.08, assuming it were established, by using evidence to establish secondary considerations, such as commercial success, long-felt but unsolved needs, and failure of others. *Graham v. John Deere Co.*, 383 U.S. at 17, 148 USPQ at 467.

Applicants submit that there was a long-felt but unsolved need for human antibodies against human HLA-DR. Anti-HLA-DR antibodies had been envisaged for a number of years as therapeutic candidates prior to the filing date of the present application. Indeed, in a series of studies dated as early as 1983, mouse anti-HLA-DR antibodies had been shown to have beneficial effects in several mouse disease models. See Waldor et al., 1983; Jonker et al., 1988; Stevens et al., 1990; Smith et al., 1994; Vidovic & Torral, 1998; Vidovic & Laus, 2000. However, mouse antibodies are unsatisfactory for clinical use due to their unwanted immunogenicity in human. Humanized antibodies had been proposed as an alternative to mouse antibodies, but they have their own limitations. First, the humanization procedure often detrimentally affects the affinity and/or specificity of the desired mouse antibody. See Slavin-Chiorini et al., Cancer Biother Radiopharm 12: 305-316 (1997). Second, once a mouse antibody has been humanized, it is very difficult to optimize its specificity or affinity. Given the limitations of mouse antibodies and humanized antibodies against human HLA-DR, human

antibodies had been highly desirable. Yet, prior to the filing date of the present application, no anti-HLA-DR antibody of human composition had been described that displayed the recited biological properties of cytotoxicity, specificity and affinity.

Applicants have filled the long-felt need by producing anti-HLA-DR human antibodies and fragments. Because these are antibodies of human composition, they are unlikely to cause immunogenicity problems for human therapeutic uses. Furthermore, Applicants have shown that these human anti-HLA-DR antibodies have surprisingly superior properties as compared to antibodies disclosed in the references. First, these human antibodies have higher binding affinities for the human HLA-DR, as described above in Example 9 of the present application. Second, several human antibodies of the instant application have significantly improved killing efficiency, as compared to two commonly used murine antibodies against human HLA-DR, 8D1 and L243. For example, the IgG forms of certain antibody fragments of the invention showed approximately 1.5 orders of magnitude improvement in EC50 compared to 8D1 and L243. See Table 6 and Figure 7.

Applicants' significant contribution to the art was recognized by the scientific community. After the filing date of the present application, this work was published in Nature Medicine, a prestigious peer-reviewed journal. See Nagy et al., Nature Medicine, 8: 801-7 (2002), a copy of which is attached as Exhibit 2. Moreover, Applicants' work was published with a concurrent News and Views article, a prestige usually reserved for important discoveries. The News and Views article, entitled DR's Orders: Human Antibody Kills Tumors by Direct Signaling, was authored by Dr. Dan L. Longo, Chief of Lymphocyte Cell Biology Unit at National Institute on Aging, and an expert in the field of immunology. See D. Longo, Nature Medicine 8: 781-3 (2002) ("Longo"), a copy of which is enclosed as Exhibit 3. In the article, Dr. Longo praised the present work as an "important step toward more effective therapies". See Longo, at 782. Recognizing that the production of desired human antibodies was no easy task, Dr. Longo concluded that "[t]he steps that Nagy et al. used in the construction of the antibody are easy to relate and <u>difficult</u> to accomplish." See Longo, at 782 (emphasis added). The recognition by the scientific community attests to the non-obviousness of the present invention.

For reasons presented above, Applicants submit that claims 1-5, 66 and 68 are non-obvious over the references cited. Nevertheless, solely to expedite prosecution of the remaining

claims, Applicants hereby cancel claims 1-5, 66 and 68. Applicants reserve the right to pursue claims of similar scope in a future application.

10. Claim 1 is additionally rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over any of Ishizuka et al., or Eray et al., or Nakamura et al., or Kita et al., or Funakoshi et al., in view of Scholm, Winter et al., Engberg et al. and Ames et al. Applicants traverse this rejection for the same reason described in section 9 above. Nevertheless, solely to expedite prosecution of the remaining claims, Applicants hereby cancel claim 1, and reserve the right to pursue claims of similar scope in a future application.

11. Claims 6-11, 18-20, 30-37, 55-56, 59-61, 71-78 and 88-95 are rejected under 35 U.S.C. 102(b) for allegedly being anticipated by Nagy et al., as evidenced by Drenou et al. and Abbas et al, and additionally rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Nagy in view of several other references. Applicants traverse these rejections to the extent it is maintained in light of the claim amendments. These claims are dependent on either claims 1-5 or claims 66-68. As argued above, claims 1-5 and 66-68 are neither anticipated by Nagy, nor unpatentable over the combination of Nagy and other references cited by the Examiner. Because a dependent claim incorporates every element of the independent claim that it depends from, the respective dependent claims of claims 1-5, and 66-68 are thus neither anticipated by Nagy, nor unpatentable over the combination of Nagy and other references cited by the Examiner as well.

Nevertheless, solely to expedite prosecution, Applicants have amended claims 6-11, 18-20, 33-36, 55-56, and 59-61 to be dependent upon allowable claims 22-29, and amended claims 71-78 and 92-95 to be dependent upon allowable claims 67 and 80-87. As dependent claims, these amended claims incorporate every element of the allowable base claims, and are thus novel and non-obvious over prior art as well. Applicants submit that support for these dependent claims can be found throughout the application. In addition, solely to expedite prosecution, Applicants have cancelled claims 30-32 and 88-91, and reserve the right to pursue claims of similar scope in a future application.

12. Claim 69 is rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Nagy in view of several other references. Applicants traverse this rejection to the extent it is maintained in light of the claim amendment. Claim 69 depends from claim 68. As argued above, claim 68 is not unpatentable over the combination of Nagy and other references cited by the Examiner. Because a dependent claim incorporates every element of the independent claim that it depends from, the dependent claim of claim 68 is thus not unpatentable over the combination of Nagy and other references cited by the Examiner as well.

Nevertheless, solely to expedite prosecution of the remaining claims, Applicants hereby cancel claim 69, and reserve the right to pursue claims of similar scope in a future application.

13. Applicants add new claims 126-129, which are directed to human IgG antibodies with combinations of VH and VL domains found in one of clones disclosed in the instant application. The specification provides ample support for claims 126-129. For example, Example 5 of the specification, entitled "Generation of IgG", disclosed the procedure for deriving IgG antibodies from these clones (see pages 51-53 of the specification). No new matter has been entered. Applicants submit that claims 126-129 are in condition for allowance.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner is invited to contact the undersigned at 617-951-7000. A petition for a three-month extension of time, with authorization to charge the required fee to Deposit Account No. 18-1945, Order No. GPCG-P01-003, is being filed concurrently. If a further extension is required, Applicants' attorney respectfully requests that such extension be granted and any fee required be charged to Deposit Account No. 18-1945, Order No. GPCG-P01-003.

Respectfully Submitted,

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Weishi Li
Reg. No. 53, 217

Customer No: 28120
Docketing Specialist
Ropes & Gray
One International Place
Boston, MA 02110
Phone: 617-951-7000
Fax: 617-951-7050

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Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells

ZOLTAN A. NAGY[1], BERND HUBNER[2], CORINNA LÖHNING[2], ROBERT RAUCHENBERGER[2], SILKE REIFFERT[2], ELISABETH THOMASSEN-WOLF[2], STEFAN ZAHN[2], SIGMAR LEYER[1], EVA M. SCHIER[1], ANGELIKA ZAHRADNIK[1], CHRISTOPH BRUNNER[1], KURT LOBENWEIN[1], BENNO RATTEL[1], MICHAEL STANGLMAIER[3], MICHAEL HALLEK[3], MARK WING[4], STEVE ANDERSON[5], MATT DUNN[5], TITUS KRETZSCHMAR[2] & MICHAEL TESAR[2]

[1]*GPC Biotech AG, Martinsried/Munich, Germany*
[2]*MorphoSys AG, Martinsried/Munich, Germany*
[3]*Medical Clinic III, Univ. Clinic Grosshadern, Munich, Germany*
[4]*Huntingdon Life Sciences, Huntingdon, England*
[5]*GPC Biotech Inc., Waltham, Massachusetts, USA*
B.H.. C.L., R.R. and S.R. contributed equally to this study.
Correspondence should be addressed to Z.A.N.; email: zoltan.nagy@gpc-biotech.com

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The Human Combinatorial Antibody Library (HuCAL) was screened for antibodies specific to human leukocyte antigen-DR (HLA-DR) that induce programmed death of lymphoma/leukemia cells expressing the target antigen. The active Fab fragments were affinity-matured, and engineered to IgG_4 antibodies of sub-nanomolar affinity. The antibodies exhibited potent *in vitro* tumoricidal activity on several lymphoma and leukemia cell lines and on chronic lymphocytic leukemia patient samples. They were also active *in vivo* in xenograft models of non-Hodgkin lymphoma. Cell death occurred rapidly, without the need for exogenous immunological effector mechanisms, and was selective to activated/tumor-transformed cells. Although the expression of HLA-DR on normal hematopoietic cells is a potential safety concern, the antibodies caused no long-lasting hematological toxicity in primates, *in vivo*. Such monoclonal antibodies offer the potential for a novel therapeutic approach to lymphoid malignancies.

Monoclonal antibodies directed against cell-surface-differentiation antigens such as CD20 and CD52 have proven useful in the treatment of lymphoid malignancies[1,2]. These antibodies exert their *in vivo* effect largely through the immunological effector mechanisms of complement-mediated lysis and/or antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, their efficacy is dependent on intact immunological mechanisms in the treated patients. The CD molecules targeted by these antibodies are widely expressed on normal lymphoid/myeloid cells[2–4] in addition to malignant cells, and thus, the therapy leads to elimination of certain normal cell populations as well as tumor cells . It would be useful to develop antibodies with an inherent capability to kill tumor cells, that is, independent of complement and ADCC, and with superior selectivity toward neoplastic cells.

Monoclonal antibodies against major histocompatibility complex class II molecules (MHC-II) offer the potential to fulfill these requirements. MHC-II molecules are noncovalently associated heterodimers of two transmembrane glycoproteins, the 35-kD α-chain and the 28-kD β-chain[5]. In humans, they occur as three highly polymorphic isotypes, termed human leukocyte antigen (HLA)-DR, HLA-DP and HLA-DQ, which under normal conditions are selectively expressed on cells of the immune system (B lymphocytes, activated T lymphocytes, macrophages and dendritic cells). Several different MHC-II-specific antibodies of murine origin have been shown to inhibit growth and induce programmed death of MHC-II-bearing tumor cells[6–10]. There is also evidence for efficacy and selectivity of murine anti-MHC-II in mouse lymphoma models, *in vivo*[11]. Although murine antibodies are unsuitable as therapeutic agents, these data indicate a potential clinical usefulness of anti-MHC-II antibodies of human composition in the therapy of lymphoma/leukemia.

Certain aspects of MHC-II-mediated cell death remain controversial. For example, the degree of selectivity is unclear: some investigators provided evidence for tumor-specificity[10–13], whereas others demonstrated effects on activated or resting normal B cells[8,9], in addition to neoplastic cells. Another controversial point is the mechanism of MHC-II-mediated cell death. Apoptosis induced directly[8,10], or via CD95 (ref. 14) or the tumor necrosis factor receptor[15] have all been implicated. However, other studies have described a non-apoptotic morphology[9], and demonstrated caspase-independence[16] of MHC-II-mediated cell death.

To generate fully human HLA-DR-specific tumoricidal antibodies with therapeutic potential, we screened the Human Combinatorial Antibody Library (HuCAL)[17] for appropriate specificities. We report here the successful identification of

Table 1 Specificity of HLA-DR-binding scFv fragments and optimized antibodies

Target protein	Reactivity to target protein														
scFv:	17	2E	45	5C	73	8A	A1	B8	E6	FD	159	170	Antibody:	1D09C3	1C7277	305D3
DR4Dw4 (purified)	+[a]	+	+	+	+	+	+	+	+	+	+	+		+	+	+
Chimeric HLA-DR–IE (purified)	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+
Lysozyme	–[b]	–	–	–	–	–	–	–	–	–	–	–		–	–	–
Transferrin	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–
BSA	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–
Human γ globulin	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–
HLA–[c]																
HLA-DR1	+[d]	+	–[e]	–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR2	+	+	–	–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR3	+	+	–	–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR4Dw4	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+/–[f]
HLA-DR4Dw10	+	+	–	+/–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR4Dw14	+	+	+	+/–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR8	+	+	–	+/–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR9	+	+	+/–	+/–	+	+	+	+	+	+	+	+		+	+	+/–
HLA-DR13	+	+	–	–	+	+	+	+	+	+	+	+		+	+	+
HLA-DR14	+	+	+	+/–	+	+	+	+	+	+	+	+		+	+	+
HLA-DRw52	+	–	–	–	n.t.	+	–	+	+	+	n.t.	n.t.		+/–	+/–	+/–
HLA-DRw53	+/–	–	+	–	n.t.	+	–	+	+/–	+/–	n.t.	n.t.		+	+	+
HLA-DPw4/w4.2	–	–	–	–	n.t.	+	–	–	–	–	n.t.	n.t.		+/–	–	+/–
HLA-DPw2/w2.1	–	–	–	–	n.t.	+/–	–	–	–	–	n.t.	n.t.		–	–	–
HLA-DQ7/w2	–	–	–	–	n.t.	+	–	+	–	–	n.t.	n.t.		–	–	–

[a], in ELISA, OD (at 370 nm – background): >1.5; [b], in ELISA, OD (at 370 nm – background): >0.5; [c], determined by FACS analysis of HLA-DR homozygous typing cell lines or L cells transfected with the respective HLA molecule; [d], mean fluorescence intensity >30; [e], mean fluorescence intensity <10; [f], mean fluorescence intensity 10–30; n.t., not tested.

candidate antibodies, and their optimization to achieve sub-nM affinity by light-chain complementarity-determining region (L-CDR) diversification. The resulting fully human antibodies were engineered as IgG_4, thereby minimizing potential side effects due to Fc-portion-mediated effector functions[18,19]. We demonstrate excellent tumoricidal activity of these antibodies both *in vitro* and in animal models, and show that they act selectively on tumor-transformed and activated cells via a non-apoptotic mechanism.

Identification of tumoricidal HLA-DR-specific antibodies

Some of the important biological activities of anti-HLA-DR such as inhibition of interaction between $CD4^+$ T cells and antigen-presenting cells, and tumoricidal activity, are associated with specificity for the first, N-terminal domains of HLA-DR molecules[20]. Because of this, we used purified HLA-DR molecules as well as human-murine chimeric MHC-II molecules (HLA-DR first domains grafted onto a murine class II molecule[21]) for screening HuCAL. A standard protein solid-phase panning[22] on purified HLA-DR molecules and a differential whole cell-panning[23] on PRIESS cells yielded 12 scFv-s that bound to HLA-DR and chimeric MHC-II molecules expressed on cells, but showed no reactivity to either $I\text{-}E^d$ (the murine part of chimeric MHC-II; ref. 21), or unrelated proteins, such as lysozyme, transferrin, bovine serum albumin and human γ globulin (Table 1), indicating that they were specific for the first domains of HLA-DR molecules. The fine specificity of scFv-s was tested on a panel of HLA-DR-homozygous typing cells and MHC-II transfectants. 10 of 12 scFv-s reacted with all major allelic forms of HLA-DR represented in the cell panel (HLA-DR1–14), and 6 of 12 recognized additional MHC-II molecules (HLA-DRw52, HLA-DRw53, HLA-DP and HLA-DQ molecules; Table 1). Thus, these antibodies could potentially be used across human populations irrespective of polymorphic differences in MHC. Most importantly, 4 of the 12 hits (17, 8A, B8 and E6) exhibited strong tumor-killing activity when cross-linked with anti-tag antibodies (data not shown). The monovalent fragments were not tumoricidal, corroborating previous observations[20].

Construction of high affinity anti-HLA-DR by CDR optimization

As the tumoricidal hits had modest affinities (for example clone B8; Table 2), they were subjected to '*in vitro* affinity maturation'. The parental scFv-s were first converted into Fab format that is less prone to aggregation and hence should give more reliable K_{off} values.

In the first round of optimization, the L-CDR3-sequences of clones 17, 8A, B8 and E6 were diversified by substituting the parental sequence with λ (17, B8 and E6) or κ (8A) light-chain repertoires from HuCAL (ref. 17). The libraries were subjected to either two rounds of solid-phase panning, or one round of solid-phase followed by whole-cell panning. To enhance panning stringency, low antigen concentrations[24,25], different concentrations of ammonium isothiocyanate[26], or increasing numbers of wash cycles[27,28] were applied. These strategies resulted in the selection of Fabs with at least two-fold better k_{off} values from the B8 library. The best clone (7BA) had a K_d of 58 nM, corresponding to a 6-fold affinity improvement compared with the best unoptimized clone B8 (Table 2). The L-CDR3-libraries from clones 17, E6 and 8A did not yield improved Fabs. We therefore focused on binders from the B8 library for further affinity improvement.

In the second round, we performed a L-CDR1 optimization. The L-CDR1 library was generated from a pool of 16 clones (including 7BA) encoding Fabs with different L-CDR3 sequences and with k_{off} values equal to or better than those of the parental molecules. Prolonged wash cycles and competing antigen enabled us to select Fabs with affinities of approximately 3 nM (Table 2). Finally, the best Fabs (305D3, 1D09C3, 1C7277, plus the unoptimized B8 for comparison) were converted into IgG_4 antibodies of subnanomolar affinity (0.3–0.6 nM; Table 2), without any major change in specificity (Table 1).

Table 2 Affinity improvements achieved by antibody optimization

Antibody	Format	Optimization	k_{on} [$s^{-1}M^{-1}$] x 10^5	k_{off} [s^{-1}] x 10^{-3}	K_d [nM]
B8	Fab	parental	0.99 ± 0.4	29 ± 8.4	346.1 ± 140.5
7BA	Fab	L-CDR3	0.96 ± 0.14	5.4 ± 0.73	58.6 ± 11.7
305D3	Fab	L-CDR3+1	1.9 ± 0.14	0.55 ± 0.05	2.96 ± 0.46
1C7277	Fab	L-CDR3+1	1.65 ± 0.21	0.44 ± 0.06	2.69 ± 0.25
1D09C3	Fab	L-CDR3+1	1.67 ± 0.16	0.49 ± 0.03	2.93 ± 0.27
305D3	IgG_4	L-CDR3+1	0.71 ± 1.6	0.33 ± 1	0.5 ± 0.2
1C7277	IgG_4	L-CDR3+1	0.71 ± 1.2	0.41 ± 1.1	0.6 ± 0.2
1D09C3	IgG_4	L-CDR3+1	0.11 ± 2	0.31 ± 0.4	0.3 ± 0.06

Tumoricidal activity of high-affinity human anti-HLA-DR

The IgG_4 antibodies were tested for induction of tumor-cell death on a panel of 24 HLA-DR$^+$ and 4 HLA-DR$^-$ cell lines, representing a variety of lymphoma/leukemia types (Table 3). The antibodies induced death in 23 of 24 HLA-DR$^+$ tumor lines. Only one line (MHH-PREB-1) was not killed despite HLA-DR expression (this exception will be described below). The viability of HLA-DR$^-$ cell lines was not significantly affected. For comparison, two murine anti-HLA-DRs that are known to induce cell death[7,10], L243 and 8D1, were tested on the same panel (at 4-fold higher concentration than the human antibodies). The murine antibodies usually killed fewer cells than the human antibodies, or failed to induce death in some HLA-DR$^+$ lines. Statistical analysis of the data in Table 3 revealed a non-linear correlation between killing efficiency and the level of HLA-DR expression, with a significantly greater killing efficiency and better correlation for the human antibodies. In direct comparisons, the human antibodies achieved 50% killing efficiency at 20–30-fold lower concentrations than the murine antibodies (data not shown). The superior performance of human antibodies could be explained, at least in part, by their higher affinity (K_d, 0.3–0.6 nM (Table 2) compared with L243, 10 nM; and 8D1, >30 nM (data not shown)).

We investigated whether the human anti-HLA-DR would also be active on freshly isolated leukemic cells. Purified malignant B cells obtained from the peripheral blood of 10 chronic lymphoid leukemia (CLL) patients[29] were efficiently and uniformly killed by the human antibodies (Fig. 1*a*). Reduction of cell viability was already observed after 4 hours of treatment, and after 24 hours only about 30–35% of cells remained viable. A control murine anti-HLA-DR without inherent tumoricidal activity[20], 10F12, had no effect on CLL cells. Under the same conditions, Rituxan (anti-CD20)[1] and Campath-1H (anti-CD52)[2] induced no detectable cell death. Apoptotic activity of these two antibodies was only detectable after cross-linking with a second antibody (anti-IgG), and remained inferior to that of noncross-linked anti-HLA-DR (data not shown).

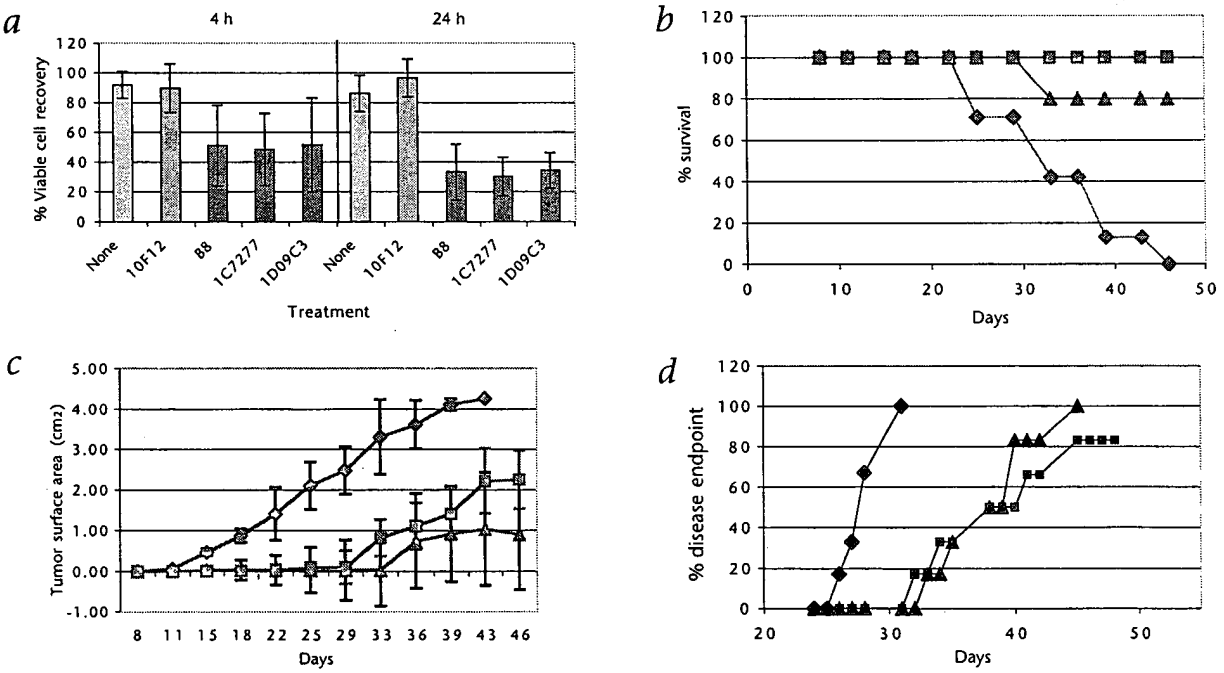

Fig. 1 *a*, Killing efficiency of anti-HLA-DR *ex vivo* against CLL cells and *in vivo* in xenograft models. Percent viable CLL-cell recovery determined by fluorescence microscopy (average ± s.d. from 10 patients, without selection for HLA-DR type) after 4- or 24-hour incubation with 100 nM of specified antibody, compared with untreated cells. *b*, Survival of SCID mice injected s.c. with GRANTA-519 cells. Antibody 1D09C3 was given at 3 x 1 mg/mouse on days 5, 7 and 9 s.c. or i.v. $n = 7$ (control) or 5 (antibody-treated) mice per group. ◆, control (solvent-treated); ■, antibody treatment s.c.; ▲, antibody treatment i.v. *c*, Effect of antibody on subcutaneous tumor growth. Same experiment as in *a*. *d*, Effect of antibody on disease incidence in SCID mice injected i.v. with GRANTA-519. Antibody 1D09C3 was administered i.v. as above. $n = 6$ mice per group. ◆, control (solvent-treated); ■, antibody 3 x 1mg; ▲, antibody 3 x 0.2 mg.

Table 3 Killing of lymphoid tumor cell lines by anti-HLA-DR

Cell line		HLA-DR	% killing by antibody[b]				
		Expression[a]	Murine antibodies (200 nM)		Human antibodies (50 nM)		
Name	Tumor type		L243	8D1	1D09C3	1C7277	305D3
LG-2	B-lymphoblastoid	458	79	85	87	88	82
PRIESS	B-lymphoblastoid	621	83	50	88	93	74
ARH-77	B-lymphoblastoid	301	88	73	85	88	87
GRANTA-519	B-cell non-Hodgkin	1465	83	56	78	78	73
KARPAS-422	B-cell non-Hodgkin	211	25	32	66	68	71
KARPAS-299	T-cell non-Hodgkin	798	78	25	82	79	76
DOHH-2	B-cell lymphoma	444	29	23	59	60	53
SR-786	T-cell lymphoma	142	3	8	53	44	26
MHH-CALL-4	B-ALL	348	35	41	63	46	43
MN-60	B-ALL	1120	46	22	69	66	67
BJAB	Burkitt lymphoma	338	53	59	71	67	64
RAJI	Burkitt lymphoma	617	69	64	84	86	83
L-428	Hodgkin lymphoma	244	82	81	91	91	92
HDML-2	Hodgkin lymphoma	326	77	73	88	84	90
HD-MY-Z	Hodgkin lymphoma	79	35	39	69	57	72
KM-H2	Hodgkin lymphoma	619	81	56	86	88	87
L1236	Hodgkin lymphoma	41	52	62	63	66	66
BONNA-12	Hairy cell leukemia	2431	92	91	92	91	86
HC-1	Hairy cell leukemia	372	88	89	93	86	93
NALM-1	CML	1078	44	4	82	78	65
L-363	Plasma cell leukemia	49	6	5	26	24	19
EOL-1	AML (eosinophil)	536	22	13	69	49	53
LP-1	multiple myeloma	315	12	0	73	70	73
RPMI-8226	Multiple myeloma	19	6	0	29	26	19
MHH-PREB-1	B-cell non-Hodgkin	175	3	3	4	8	11
OPM-2	Multiple myeloma	3	13	0	1	4	5
KASUMI-1	AML	5	0	0	10	10	6
HL-60	AML	3	18	0	15	9	22
LAMA-84	CML	7	7	9	11	5	7

[a], Expressed as mean fluorescence intensity after staining with FITC-labeled L243 antibody. Numbers are the mean of 1-3 experiments per cell line. [b], Based on viable cell recoveries determined by light or fluorescent microscopic cell counting or FACS analysis, as described in Methods. Each number represents the average of 2–6 independent experiments.

To test the *in vivo* efficacy of anti-HLA-DR, we inoculated severe combined immunodeficient (SCID) mice subcutaneously (s.c.) or intraveneously (i.v.) with the non-Hodgkin B-cell lymphoma line GRANTA-519 (Table 3), and monitored tumor development in antibody-treated mice in comparison to solvent-treated animals. In the s.c. tumor experiment, all control mice died within the observation period, whereas all but one antibody-treated mouse survived (Fig. 1*b*). Tumor growth was also significantly retarded in the treated animals (Fig. 1*c*). In the i.v. tumor model, a significant delay in disease onset was observed in the antibody-treated groups (Fig. 1*d*). Altogether, these efficacy data are promising, and raise the possibility that such antibodies could become useful and potent therapeutic agents for the treatment of different HLA-DR$^+$ lymphomas and leukemias.

Mechanism and selectivity of anti-HLA-DR-induced cell death

Conflicting reports in the literature[8–10,14–16] led us to determine whether the cell death induced by our human anti-HLA-DR is apoptotic. We used Annexin-V-FITC/propidium iodide (PI) double staining to distinguish between apoptotic and non-apoptotic (necrotic, 'dead') cells. Anti-HLA-DR induced cell death was compared with apoptosis induced by anti-CD95 antibody in the same cell line. The proportion of dead (Annexin-V$^+$/PI$^+$) cells increased far more rapidly in the anti-HLA-DR-treated cultures than among the anti-CD95-treated cells, whereas the proportion of apoptotic (Annexin-V$^+$/PI$^-$) cells increased rapidly in anti-CD95-treated cultures and not significantly in the anti-HLA-DR-treated ones (Fig. 2*a* and *b*). We also attempted to inhibit cell death with zDEVD-fmk, an irreversible caspase-3 inhibitor, and zVAD-fmk$_2$, a broad-spectrum caspase inhibitor. Cell death induced by anti-HLA-DR was not affected, whereas CD95-induced death in the same cell line was significantly reduced by caspase inhibitors (data not shown). Consistent with the lack of caspase-3 activation, poly(ADP-ribose) polymerase (PARP) was not cleaved in anti-HLA-DR-treated cells (data not shown). Similar results were reported by others using murine anti-HLA-DR (ref. 16). Finally, we could not detect chromatin condensation typical of apoptosis in anti-HLA-DR-treated cells. We therefore concluded that the cell death induced by the human anti-HLA-DR does not occur via the classical apoptotic pathway.

Because MHC-II molecules are constitutively expressed on B lymphocytes, the most obvious side effect of anti-HLA-DR treatment would be the killing of normal B cells. We therefore investigated the effect of our antibodies on normal human B cells. The viability of purified resting B cells was not significantly altered by human anti-HLA-DR (Fig. 2*c*). In contrast, pokeweed mitogen–activated B cells from the same donor were killed by these antibodies. No death of either inactivated or activated B cells was induced by the control antibody 10F12. Similar results were obtained with resting and lipopolysaccharide-stimulated splenic B cells from HLA-DR-transgenic mice (data not shown). Thus, it seems that the antibodies can kill activated but not resting MHC-II$^+$ normal cells in addition to tumor cells, suggesting a dual requirement of both MHC-II expression and cell activation for antibody-induced death. Because the vast majority (up to 99%) of peripheral B cells are resting, the potential side effect

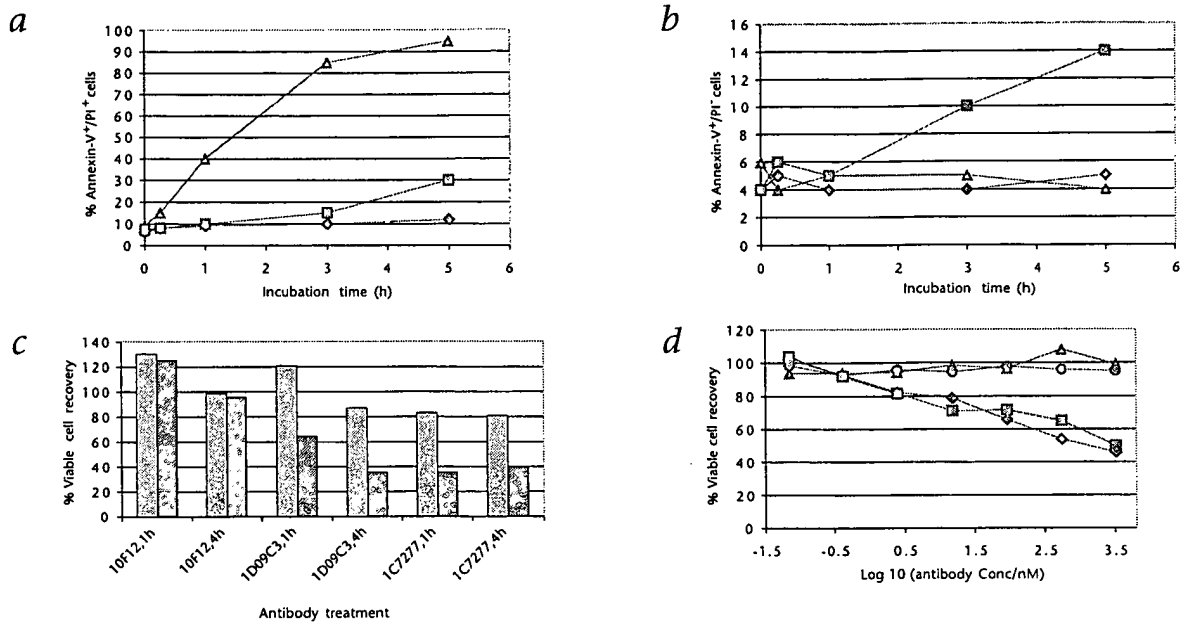

Fig. 2 Mechanism and selectivity of anti-HLA-DR-induced cell death. *a*, Comparison of death induced in PRIESS cells by the Fab fragment of human anti-HLA-DR antibody B8, crosslinked with anti-FLAG and anti-CD95, respectively. ◆, anti-FLAG; ■, anti-CD95; ▲, B8-Fab+anti-FLAG. *b*, Comparison of apoptosis induced in PRIESS cells after anti-HLA-DR and anti-CD95 treatment (as in *a*). Percent positive cells was determined by FACS analysis. *c*, Activated but not resting normal human B cells are killed by anti-HLA-DR treatment. B cells isolated from PBL by magnetic sorting were treated with 50 nM of different antibodies (inactivated), or stimulated with pokeweed mitogen for 3 d (activated) and subsequently treated with antibodies . ▨, resting; ■, activated. *d*, The lymphoma line MHH-PREB-1 becomes susceptible to anti-HLA-DR-mediated death after activation with interferon-γ and lipopolysaccharide. Viable cell recovery is based on duplicate determinations with s.d. < 10%, and expressed as % of untreated controls. ◆, 1C7277-treated activated; ▩, 1D09C3-treated activated; ▲, 1C7277-treated inactivated; ●, 1D09C3-treated inactivated.

due to killing of normal B cells is anticipated to be negligible.

The cell line MHH-PREB-1 was resistant to killing despite HLA-DR-expression, possibly because it was not activated (Table 3). We therefore stimulated these cells with interferon-γ and lipopolysaccharide. Activation was evidenced by an increase in the cell-surface expression of CD40 and HLA-DR. The activated tumor line became susceptible to antibody-induced cell killing (Fig. 2*d*). It is unlikely that the susceptibility to killing was solely a consequence of increased HLA-DR expression, because several different cell lines are readily killed, although they express lower levels of HLA-DR than inactivated MHH-PREB-1 (Table 3). It remains to be determined what specific steps of activation are necessary to undergo cell death. Cell proliferation is apparently not needed, as tumor cells in mitosis arrest remain susceptible to antibody-mediated killing (data not shown).

Reversible hematological effects of anti-HLA-DR in primates

As certain hematopoietic stem cells express HLA-DR (refs. 30,31), we investigated possible hematological effects of antibody 1D09C3 in cynomologus monkeys. Cynomologus is a relevant species for toxicity studies, because the antibody cross-reacts with its HLA-DR molecules, and the tissue distribution of antibody reactivity is identical to that of humans (data not shown). Three animals were dosed with 1 mg/kg 1D09C3 antibody i.v. on day 1, followed by 10 mg/kg i.v. on day 8, and hematology was monitored from day −7 until day 16. Clinical chemistry was done on day 1 pre-dose and on day 12. On day 16, complete macroscopic pathology and histopathology were performed on the animals.

The treatment was well tolerated, without clinical symptoms. Blood lymphoid-cell counts were affected by the treatments to differing extents (Fig. 3). The lower dose of antibody (insufficient to saturate all HLA-DR sites on blood lymphoid cells) caused a rapid decrease in T, B and natural killer (NK) cells with recovery within 24 hours, suggesting cell margination rather than genuine depletion. A transient increase in monocyte numbers was also evident, returning to normal level in 48 hours. At the higher dose (saturating all HLA-DR sites on

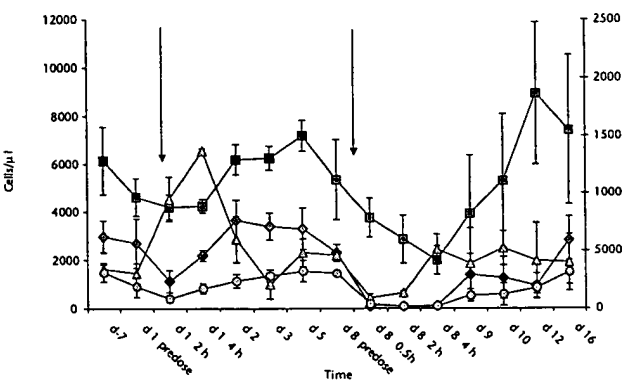

Fig. 3 Changes in blood lymphoid-cell counts of Cynomologus monkeys after treatment with antibody 1D09C3. Left ordinate: ◆, B lymphocytes (CD20⁺); ■, T lymphocytes (CD3⁺). Right ordinate: ▵, monocytes (CD14⁺); ◎, NK cells (CD3-CD56⁺). Arrows indicate time-points of treatment. Average counts ± s.d. from 3 animals are shown.

B cells and monocytes), a profound depletion of B cells was observed, with full recovery taking up to 8 days. T cells were also significantly depleted but recovered after 48 hours, with a subsequent rebound. NK-cell depletion also occurred with full recovery in 4 days. After both treatments, a rapidly reversible (within 24 hours) increase of neutrophil and decrease of eosinophil and basophil leukocyte counts were detected (data not shown). The full recovery from hematological changes, particularly after high-dose antibody, indicated that no irreversible inhibition of hematopoiesis occurred. No pathological changes were observed either macroscopically or histologically. The increased cell numbers seen in the cortex and paracortex of lymph nodes and peri-arteriolar lymphoid sheaths of the spleen was consistent with the observed T-cell rebound. In bone-marrow smears, a marginally increased myeloid:erythroid cell ratio was observed, consistent with slightly accelerated myelopoiesis (possibly to replenish depleted B cells). Clinical chemistry was negative. The overall favorable side-effect profile encouraged us to enter further preclinical studies with this antibody.

Discussion

There are two additional anti-HLA-DR with therapeutic potential, Lym-1 (refs. 12,32) and 1D10 (ref. 13), which differ in many respects from the antibodies we describe here. These two antibodies recognize what seem to be post-translational modifications on HLA-DR molecules that occur preferentially in B cell–derived tumors, thus providing a margin of selectivity, although some expression was also noted on normal B cells and monocytes[12,33]. Neither of these antibodies has inherent tumoricidal activity, and thus, Lym-1 is developed in a ^{131}I-labelled form (Oncolym), whereas the efficacy of 1D10 relies on intact immunological effector mechanisms of the patient, similarly to other antibodies[1,2] already available for the clinic. Furthermore, Lym-1 is a murine antibody with substantial immunogenicity for humans, and 1D10 is a humanized antibody. Our fully human antibodies with strong inherent tumoricidal activity and selectivity for activated and/or tumor-transformed cells demonstrate a substantially different profile and mechanism of action from these two antibodies, and thus promise a novel therapeutic approach to lymphoma/leukemia.

Methods

Cell lines. Tumor-cell lines were purchased from DSMZ (Braunschweig, Germany), and ECACC (Salisbury, UK). Hybridoma L243 was from ATCC (Rockville, Maryland). Human B cells were purified from peripheral blood by magnetic-bead sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany). CLL cells were isolated from blood samples of B-CLL patients as described[29]. All CLL samples showed strong HLA-DR expression (mean fluorescence intensity 123-865 by FACS analysis using FITC-L243).

Preparation of HLA-DR molecules. The HLA-DR molecule DRA*0101/DRB1*0401was prepared from the homozygous B-lymphoblastoid line PRIESS, and the human–mouse chimeric molecule from transfectant M12.C3.25 (ref. 21, a gift from K. Ito) by affinity purification[34].

Screening of HuCAL for HLA-DR-specific single-chain antibodies. The single-chain Fv-based HuCAL (ref. 17) was used for standard, manual solid-phase panning on purified HLA-DR molecules in combination with whole cell-panning on PRIESS cells as described[23].

Construction of L-CDR3 and L-CDR1 libraries. ScFv were converted into Fab following the standard conversion protocol[22] for the modular HuCAL-library. Based on parental Fabs 17, B8, E6 (all H2, λ1) and 8A (H3, κ3) a sub-library was constructed with different L-CDR3-cassettes[17]. Library sizes were 5.1–6.0 × 10^6 for λ and 1.7 × 10^6 for κ clones. L-CDR1-libraries were generated from 16 pooled Fab clones after L-CDR3 optimization. Cassettes for the L-CDR1 maturation were based on oligonucleotides synthesized of O-methyl trinucleotide phosphoramidites[35] as described[17]. The L-CDR1 (λ -1-framework) comprised two oligonucleotides encoding the following amino acids: 1) SGS(19)S(80%N/10%D,K)IG(19)(19)(19)V(19); and 2) (50%S,T)GS(80%S/20%N)SNIG(19)(19)(19)(19)V(19); from N- to C-termini; randomized residues and their ratios are in parentheses; "(19)" = all amino acids randomized except cysteine). Cassettes were introduced into a promoterless derivative of pMORPH4 (data not shown), and subsequently inserted into Fab clones[17]. The size of L-CDR1 library was ~4.2 × 10^8 c.f.u.

Selection of high-affinity Fab fragments. After L-CDR3-optimization, phage-antibodies were obtained by solid-phase-panning on 250 or 500 ng purified HLA–DR per well. Purified HLA-DR were pooled and panned on either 12 ng or 250 ng/well HLA-DR followed by 30 min incubation in 500 mM ammonium isothiocyanate between the washing and the elution step[22]. Alternatively, the second panning was performed on different numbers (1 × 10^1 to 10^5) of PRIESS cells[23]. Fab-clones were selected by k_{off} ranking. The L-CDR1-optimized pool-library was subjected to 3 manual solid-phase pannings[22] on 250 ng per well HLA-DR, combined with longer washes (6–24 × 30 min) and with 500 nM competing antigen in the wash buffer.

Affinity-determination and K_{off}-rankings. Affinities of Fabs purified from periplasmic extracts[22] by *Strep*-Tactin affinity chromatography (IBA, Göttingen, Germany) were determined using Biacore 2000. For K_{off}-ranking, periplasmic extracts were applied directly to CM5 sensor chips coupled with 10,000 resonance unit (RU)–purified HLA-DR using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC)– N-hydroxysuccinimide (NHS) coupling chemistry. Antibody affinity data are based on measuring at least 3 different preparations at 7 concentrations on CM5-chips coated with different concentrations (500–4,000 RU) of HLA-DR. Fabs for affinity determination were 100% monomeric.

IgG conversion and production. IgG_4-conversions were performed as described[22]. Antibodies were purified by Protein A Sepharose FF from supernatants of stably transfected CHO-S cells cultured in serum-free medium in 36-liter stirred tank fermentors. Antibody 8D1 was a gift of M. Sandor.

Cell killing assays. Cells (2 × 10^6/ml) were incubated in RPMI 1640 plus 2.5% FBS (Biowhittaker Europe, Belgium) and different concentrations of anti-HLA-DR at 37 °C for 4 h (or 24 h). Controls were without antibody or with murine anti-HLA-DR 10F12 that fails to induce cell death[20]. Cultures were set up in duplicate in flat-bottom 96-well plates. Because dead cells disintegrate quickly (within 30 min), percent killing was calculated based on viable cell recovery: (viable untreated – viable treated / viable untreated) × 100. Viable and dead cells were distinguished by Trypan blue for light microscopy, fluorescein diacetate (FDA; 100 μg/ml; live cells) and propidium iodide (PI, 40 μg/ml; dead cells) for fluorescent microscopy, and PI for FACS analysis. To obtain absolute cell counts by FACS, cultures were supplemented with FACS 'Truecount' beads. Cell counts were calculated by the following equation: viable cells × total beads / counted beads. The 3 different methods of cell counting yielded comparable results.

Xenotransplant models of non-Hodgkin lymphoma. 8-wk-old female C.B.-17 *SCID* mice were injected with anti-asialoGM1 antibody (Wako Chemicals, Neuss, Germany; 25 μl diluted 4-fold in PBS, i v.) to suppress natural killer–cell activity, on days 0, 1 and 2. On day one, 5 × 10^6 GRANTA-519 cells were injected s.c. into the right flank or i.v. Endpoint in the s.c. model was a tumor surface area of >5 cm^2, skin ulceration or death, and in the i.v. model hind-leg paralysis or death. Mice were treated with 1D09C3 antibody s.c. or i.v. on days 5, 7 and 9. Control mice received PBS.

Lymphoid-cell phenotyping in the blood of Cynomologus. Lymphoid cell types were differentiated by anti-human-CD combinations (BD Pharmingen Ltd) as follows: CD3-FITC/CD20-PE for T and B cells, CD3-FITC/CD56-PE for NK cells, and CD14-PC5 for monocytes. Samples were analyzed in a Beckman Coulter EPICS XL-MCL flow cytometer. To obtain absolute cell counts, 100 μl of Flow-Count (Beckman Coulter) beads were added to each sample.

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Competing interests statement

The authors declare competing financial interests: see the website (http://medicine.nature.com) for details.

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Although this new information suggests that hypothesis-driven efforts at obesity drug development can pay major dividends, the conclusion that a therapeutic breakthrough is at hand is premature. Regardless of their initial promise, most drugs fall by the wayside at some point along the tortuous path from pre-clinical promise to clinical utility. Of obvious concern is the possibility that MCH receptor blockade will have adverse effects in humans not detected in animal studies. Indeed, the hypothesis that MCH participates in regulation of food intake, mood or anxiety in humans remains untested, so the therapeutic potential of MCH-receptor antagonists in human obesity or psychiatric disorders is difficult to estimate. These concerns are allayed to some extent by evidence that key molecules governing energy homeostasis are conserved across mammalian species[5], but the hard work of translating basic findings into clinical practice still lies ahead. With the emergence of obesity as a leading cause of illness and death on a global basis[1], the urgency of this quest could not be greater.

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Department of Medicine
Harborview Medical Center and
University of Washington
Seattle, Washington, USA
Email: mschwart@u.washington.edu

DR's orders: Human antibody kills tumors by direct signaling

Murine antibodies that block cell-surface antigens have been engineered (humanized) to successfully elicit human immune-effector mechanisms. Now, a fully human antibody produced entirely *in vitro* kills tumor cells directly through signal transduction and shows promise against lymphoma in animal studies (pages 801–807).

DAN L. LONGO

In this issue, Nagy *et al.* describe the generation *in vitro* of an entirely human antibody with subnanomolar affinity for human class II MHC molecules that induces human lymphoma cell death. And the cell death occurs independent of human immune effector mechanisms[1].

They generated the targeting molecule in the laboratory using techniques that reproduce what happens inside a lymph node germinal center, where antibody genes are mutated to make an antibody that binds more tightly to an antigen. The new antibody may be even better than anything a person could generate in his or her body. If its current experimental promise is fulfilled in the clinic, the new antibody has the potential to outshine current biologically based therapies for non-Hodgkin lymphomas and, when combined with other active agents, may increase the fraction of patients cured of the disease.

Monoclonal antibody technology has revolutionized science over the last 27 years; however, the clinical benefit has been slower to emerge. But we have gradually learned how to overcome the primary barriers to putting murine monoclonal antibodies to use in people[2]. First, the killing problem: murine antibodies are not effective in killing human tumor cells in people. Second, the immunogenicity problem: murine antibodies elicit a host immune response that limits efficacy. Third, the target problem: the antibody target should be a cell-surface structure that is not shed into the serum or modulated through internalization. A tumor that can escape by no longer expressing the target will do so.

Several approaches have been taken to address these problems. Murine antibodies have been armed with radionuclides, toxins and cytotoxic drugs that are intended to kill the targeted cell. Several such products are now FDA-approved: yttrium-90-labeled anti-CD20 (ibritumomab tiuxetan, called Zevalin) and calicheamycin-conjugated anti-CD33 (gemtuzumab zogamicin, called Mylotarg). But the addition of killing molecules to the antibodies compromises their target specificity, as the toxic moiety adds its own mechanisms of cell penetration and killing to those of the antibody. The result is often serious side effects. Furthermore, these agents usually maintain or have increased immunogenicity over the non-modified antibody.

A more effective solution to the killing problem has been to generate monoclonal antibodies containing effector sequences of human origin. Genetic engineering has produced antibodies with murine-generated and *in vivo* affinity-matured antigen binding sites imbedded in antibodies with human Fc (constant) regions. Three such engineered chimeric antibodies (called humanized antibodies) have been FDA-approved for cancer: humanized anti-CD20 antibody (rituximab, called Rituxan), humanized anti-CD52 antibody (alemtuzumab called Campath) and a humanized anti-HER-2/neu antibody (trastuzumab, called Herceptin). They kill tumor cells *in vivo* using human immune effector functions such as fixation of complement (the cascade of proteins that punches

holes in cell membranes and lyses cells), and antibody-dependent cellular cytotoxicity. In the latter mechanism, the Fc portion of the antibody attracts cells that can kill the cell to which the antibody is bound. Rituximab, prescribed most often for B-cell lymphomas, kills tumor cells more effectively than a previous-generation murine antibody against the same target. However, this Fc-mediated efficacy also brings with it toxicities to the patient. Serious shortness of breath can accompany the initial infusion, via activation of immune effector mechanisms in the lung. Slowing the infusion usually reduces the problem and subsequent infusions are usually uneventful.

Antibodies of human origin also solve the immunogenicity problem. Rituximab, alemtuzumab and trastuzumab appear to be poorly immunogenic, at least in people with cancer. Rituximab is now being used to treat patients with autoimmune disorders mediated by autoantibodies, such as idiopathic thrombocytopenia, and it seems that the host rarely if ever makes antibodies directed against the rituximab.

The generation of monoclonal antibodies of fully human sequence is an even more important step toward more effective therapies. Nagy *et al.* generated a fully human antibody of IgG4 isotype, a class that is relatively devoid of Fc-mediated side effects. As their target, they chose the human leukocyte antigen (HLA)-DR molecule. HLA-DR is one of three highly polymorphic genes of the class II major histocompatibility complex (MHC-II), which, under normal conditions, are selectively expressed on cells of the immune system.

The relatively limited expression of HLA-DR on normal cells facilitates the targeting of malignant lymphoma cells that express HLA-DR. However, this target has another important advantage over other targets. Preclinical evidence is strong that additional mechanisms of killing can be brought to bear by delivering agonistic, stimulatory signals through receptors involved in cell activation and growth—such as HLA-DR on B cells. Both B-cell tumors[3,4] and T-cell tumors[5] can be killed *in vitro* and *in vivo* by signals delivered through antigen receptors. Similarly, agonistic antibodies against CD40 can kill B-cell tumors[6] and agonistic antibodies against CD30 can kill CD30$^+$ anaplastic large-cell lymphomas *in vitro* and *in vivo*[7]. Even some of the efficacy of rituximab may be related to effects on cell signaling[8]. The ability of signaling through MHC-II molecules to kill B-cell tumors has been known for 15 years in murine systems[9] and has been extended to human cells *in vitro*, though the details of the signaling pathway have not been fully defined (Fig. 1).

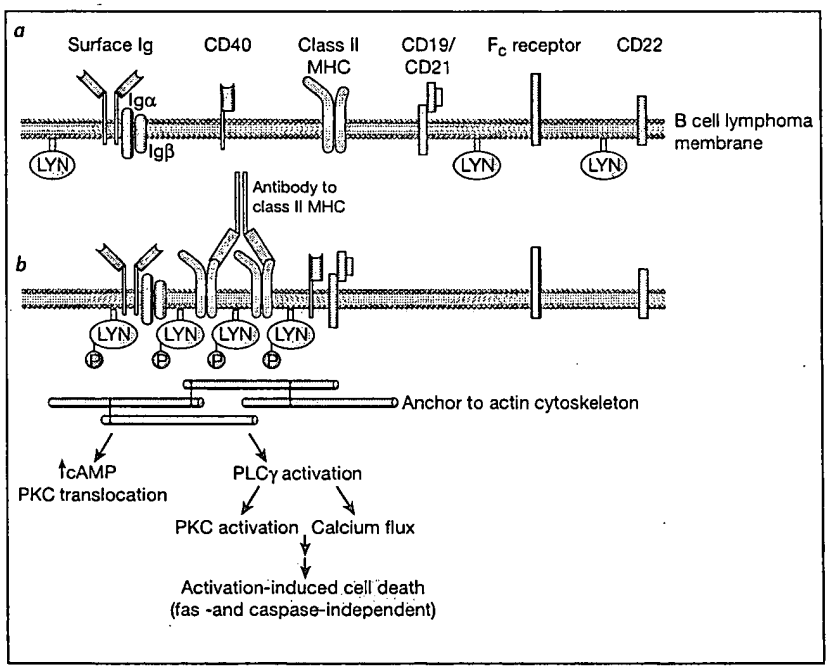

Fig. 1 Activation of cell death by monoclonal antibody signaling through class II MHC. *a*, A model of the B-cell lymphoma membrane. It contains several receptors that, when ligated, mediate cell activation, including surface IgM, CD40, MHC-II and CD19/CD21. In addition, the membrane contains receptors with negative effects on signaling such as the Fc receptor and CD22. *b*, Antibody to MHC-II brings two MHC-II molecules together and transduces a signal. The positive signaling receptors aggregate in the lipid rafts and lyn and other enzymes activated by tyrosine phosphorylation. The complex in the lipid raft is anchored to the actin cytoskeleton, and downstream signaling events activated by lyn, proceed. This leads ultimately to activation-induced cell death.

The steps that Nagy *et al.* used in the construction of the antibody are easy to relate and difficult to accomplish. The authors used a phage library containing permutations of seven human V(H) genes and seven human V(L) genes, which together account for more than 95% of human antibody diversity[10]. Phage containing single-chain Fv (the linked variable domains of the heavy and light chain) capable of binding to the human HLA-DR were isolated. Because of the tendency of Fv to self-aggregate, the single-chain Fv were converted to Fabs (the intact light chain and the variable and first constant domain of the heavy chain). Then the DR-binding Fabs were subjected to *in vitro* affinity maturation through two rounds of optimization—alteration of the hypervariable areas of the antibody binding site that form the points of contact with the antigen. First, the authors altered the hypervariable CDR3 sequences, and then the hypervariable CDR1 sequences. Finally, the Fabs with the highest affinity for DR were converted into IgG4 monoclonal antibodies. These antibodies had subnanomolar affinity (0.3–0.6 nM), which is about 10-fold stronger than most antibodies generated by immunizing humans.

In animal models, anti-MHC class II antibodies caused tumor regression. Furthermore, animals cured of lymphoma were also immune to a subsequent reintroduction of the same but not a different lymphoma. This suggests that tumor-specific immunity[9] develops as a component of the response. Furthermore, the specificity of the antibody effects in animals is noteworthy—B cells were transiently affected, as was splenic antigen-presenting function. However, Langerhans cell antigen presentation and long-term B-cell responsiveness were unaffected. The authors evalu-

ated the potential for side effects by testing the monoclonal antibody on three healthy primates. The primate studies with the new anti-DR reagent are highly reassuring that the expression of DR on a variety of non-malignant cell types, though limited, will not be clinically limiting.

Two monoclonal antibodies with specificity for some haplotypes of human DR β-chains have been used in humans and one of these, a humanized antibody called apolizumab (Remitogen) is in phase 2 clinical trials[11]. It is not clear whether either of the antibodies tested so far can mediate signal transduction. However, the reagent reported by Nagy and colleagues certainly does and is also more broadly reactive with the range of human DR polymorphisms. The promise of the new approach is that cell death is a direct consequence of antibody binding to the target on tumor cells, and it does not require additional mechanisms that may be compromised in the cancer patient. Like chemotherapeutic agents and radiation therapy, the new antibody against HLA-DR is directly cytotoxic.

Despite promising data such as these it is dangerous to make predictions about clinical success in cancer treatment. The tumor-cell lines killed by the new antibody all have the phenotype of activated B cells and have a growth fraction of 100%, which means all the cells were proliferating. The one cell line that failed to be killed could be rendered susceptible to killing by activation signals. By contrast, human lymphomas have a smaller growth fraction *in vivo* (8% or fewer of the tumor cells are actively proliferating). In humans, molecular characterization is defining distinct subgroups[12] that may or may not be susceptible to DR activation *in vivo*. However, there is every reason to think that antibodies capable of delivering signals through cell-surface receptors will be more effective than antibodies that either block function or recognize a structure that serves no functional role in the activation and growth of the tumor cell. The next step is to take the new weapon into the clinic. If it is as successful as expected, the path to the generation of other effective new treatments will be well illuminated by this elegant work.

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National Institute on Aging
Baltimore, Maryland, USA
Email: longod@grc.nia.nih.gov

Host genetics and HIV—removing the mask

A newly discovered interplay between two human genes controlling the immune response to HIV has implications for antiretroviral drug therapy and the development of preventive vaccines.

NELSON L. MICHAEL

Studies of the impact of host genetics on the susceptibility to HIV infection and rate of disease progression have revealed associations with a number of individual genes. These include those genes encoding proteins that control viral entry (CCR5, CCR2, RANTES and SDF1), immune regulation (interleukin-10, tumor necrosis factor-α and MBL) and adaptive immune recognition by T cells (human leukocyte antigen or HLA)[1,2]. Associations have not yet been described with genes that control natural killer (NK) cells of the innate immune response.

In the August issue of *Nature Genetics*, Martin *et al.* describe a complex interplay between genes that control NK cell activity and HLA genes that control immune recognition[3]. They find that the influence of the former genes masks that of the latter. This masking, or 'epistasis', represents the first such interplay between host genes to be associated with HIV disease progression. Identification of such an association advances the field to the next level in defining host genetic influences on HIV pathogenesis. This research also implicates a key interaction between the innate and adaptive arms of the cellular immune response in controlling HIV replication.

Viral pathogens are targeted by diverse immune-effector mechanisms. Cytotoxic T-lymphocytes (CTLs) represent the adaptive immune response. They control the expansion of antigen-specific cellular clones, which recognize viral antigens displayed on infected target cells by *HLA* class I gene products. Previous exposure to antigen provides for a more rapid immune response upon re-exposure. NK cells are part of the innate immune response, as previous antigen exposure does not modulate NK pathogen recognition. NK cells express cell-surface killer immunoglobulin-like receptors (KIRs) that recognize HLA class I molecules on infected target cells. KIRs can both stimulate and inhibit NK activity[4]. Thus, a mechanistically cogent but complex interplay occurs between *HLA* and *KIR* genes in the host im-